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Partial purification and characterization of soluble cyclic nucleotide phosphodiesterases in human and murine tissues

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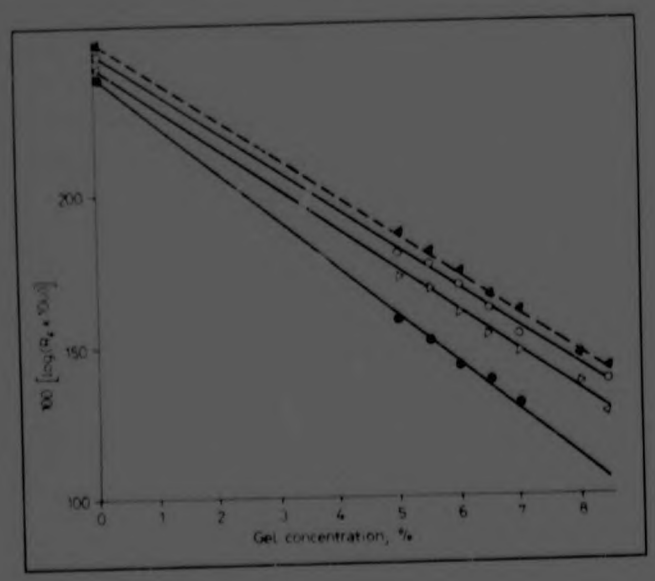
Fig. 5:3. Ferguson plot of cyclic nucleotide phosphodieterase from crude cytosol of humar leiomyoma of the uterus. Samples were run, as described in the experimental section, at different gel concentrations ranging from 5% to 8.5%. For each gel concentration, the plot of Rf (logarithmic scale) versus gel concentration (%) was constructed from the results (mean s.d. of four separate runs), by using least-squares linear regression. The plots represent band 1 (●), band 2 (△), band 3 (○) and band 6 (▲). The individual data for each band is given in Table 5:1.

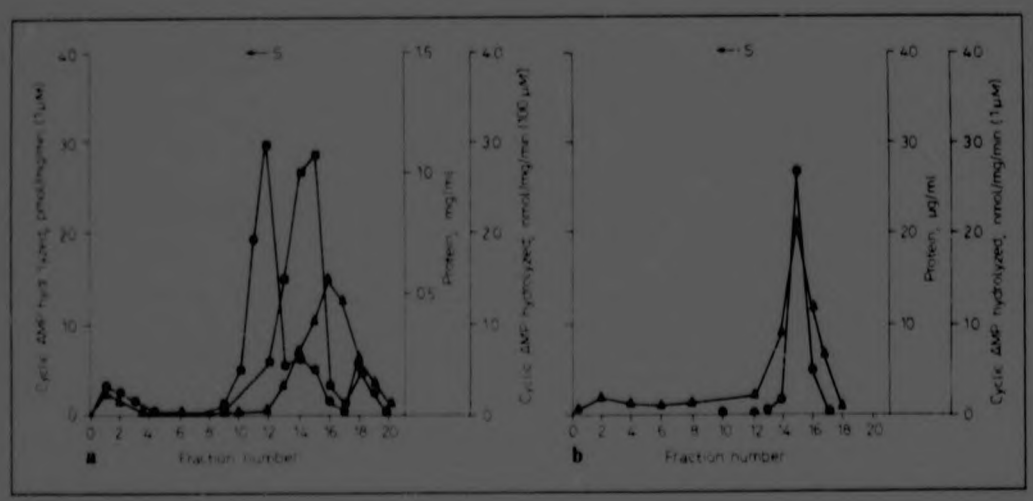


Sucrose gradient ultracentrifugation of crude leiomyoma cytosol.

This method revealed three peaks of cPDE activity (only cAMP tested) with sedimentation coefficients, calculated according to the tables of McEwan (1967), of 3.6 ± 0.35 S, 8.1 ± 0.3 S and 11.8 ± 0.3 S at 1 uM cAMP concentration (Fig. 5:4). Only two peaks at 7.9 S and 3.6 S were observed at 100 uM cAMP. The affinity purified fraction showed a single symmetrical peak of enzyme activity with 1 uM cAMP at 7.8 ± 0.3 S (Fig. 5:4). No activity could be detected at 100 uM cAMP. If one expressed the results as dpm versus fraction number, the crude cytosol also showed a peak in the identical position as that of the purified fraction. This is similar to the findings of Strada *et al.* (1981) who presented their results in the latter way although their reported sedimentation coefficient was less for rat uterus as further detailed in the discussion. It was noted that leiomyoma cytosol, which had been frozen and thawed, showed the majority of specific activity to be associated with the 3.5 S peak (result not shown).

Fig.5:4. Sucrose gradient ultracentrifugation of cPDE from human leiomyoma of the uterus.

Crude cytosol (a) (5 mg/ml protein) was loaded in 200 ul aliquots and the centrifugation and the assay of enzyme activity were performed as described in the Methods. Specific activity of cPDE was measured at 1 uM cAMP (●) and 100 uM cAMP (■). Protein (▲) was measured in mg/ml using the Bio-Rad kit. Purified isoenzymes (b), which were the bands 2-6 observed on native polyacrylamide-gel electrophoresis, were loaded in 200 ul aliquots (0.5 mg/ml protein) after affinity chromatography, ammonium sulphate precipitation and equilibration in buffer A. The enzyme activity was measured at 1 uM cAMP (●) and protein (▲) was determined by the micro-method of the Bio-Rad kit. The results shown are representative ones of three separate runs.



Soluble cPDE activities of eluted bands of leiomyoma cytosols run on non-denaturing gels.

The various activity bands of the leiomyoma were eluted and assayed for cPDE activity (only cAMP tested) and summarized below (Table 5:2)

Table 5:2. Elution of the cPDE forms from the unstained, excised cPDE activity bands of leiomyoma cytosol on 7.5% gels. The lower bands 3-6 were combined into two segments of bands 3-4 and 5-6. The effect of added calmodulin (30 units per assay) was also tested. The units are in pmol/mg protein/min.

Band No.	1 uM cAMP	SD	1uM cAMP + calmodulin	SD
1	465	58	532	72
2	782	72	830	84
3-5	1210	107	1290	116
5-6	449	32	403	33

There did not appear to be increased cPDE activity when the eluted forms were incubated in the presence of added calmodulin plus calcium (present in the assay buffer). This suggested that the calmodulin-bound cPDE forms were still bound to calmodulin after the electrophoresis and elution steps.

Kinetic properties of some of the separated forms of cPDE.

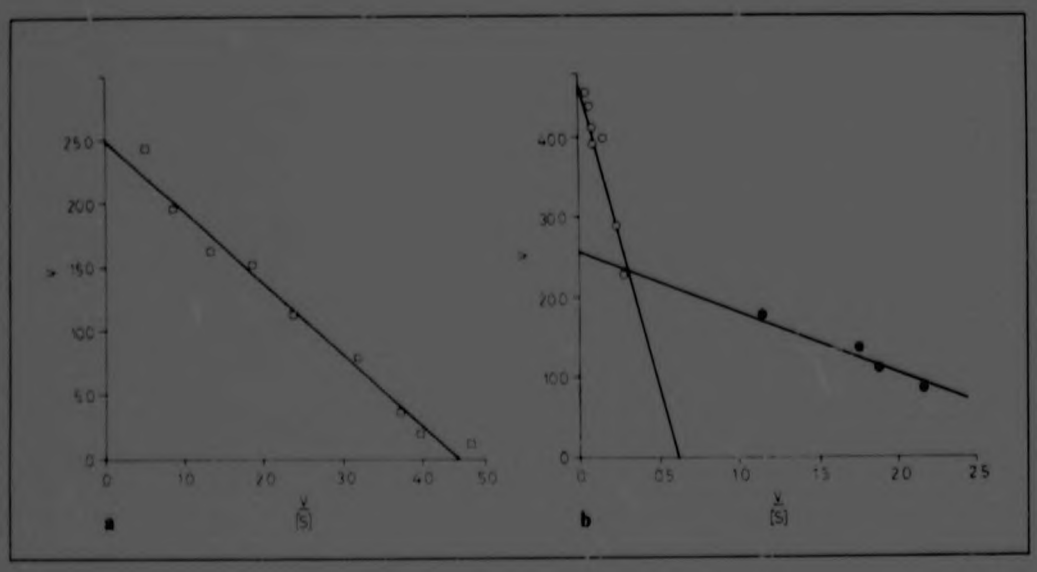
The enzyme forms, designated band 1 and 2 in polyacrylamide-gel electrophoresis (Figs. 5:1a and 5:1b) were obtained by preparative native polyacrylamide electrophoresis followed by elution of the enzyme from the gel. The Km and Vmax values for the forms were calculated from Eadie-Hofstee plots with cAMP as substrate. The plot for form 1, which displayed linear kinetics, is shown (Fig. 5:5). The Km was found to be 5 ± 0.4 uM and the Vmax 26 ± 3.5 nmol/mg/min protein. The band 2 form did not display linear kinetics (Fig. 5:5) and the data was indicative of either two enzyme forms or apparent negative co-operativity. The calculated Km values were 5.8 and 37 uM with respective Vmax values of 12 and 64 nmol/min/mg protein. The band 5 form was also studied after elution and showed non-linear kinetics with an apparent Km range of 17-24 uM and Vmax range of 85-125 nmole/min/mg protein. The anomalous kinetics may, however, have resulted from contamination of this form with those of band 3 or 4 since these shared closer Rf values and, as a result, were difficult to separate when cutting strips from preparative gels.

Table 5:3. cPDE kinetic data for bands 1,2 and 5 of human leiomyoma of the uterus.The bands were eluted from native polyacrylamide gels (7.5%) and assayed for enzyme activity between 0.5 uM and 100 uM cAMP. The data are the mean ± s.d. of three experiments except for band 5 which is expressed as a range.

Band No.	Km (uM)	s.d.	Vmax (nmol/mg/min)	s.d.
1	5.0	0.3	26	3.5
2	5.8	0.95	12	1.6
2	37.0	5.0	64	11.5
5	17-24		85-125	

It appeared that the band 1 form exhibited a higher affinity for cAMP although the Vmax for band 5 was the greatest.

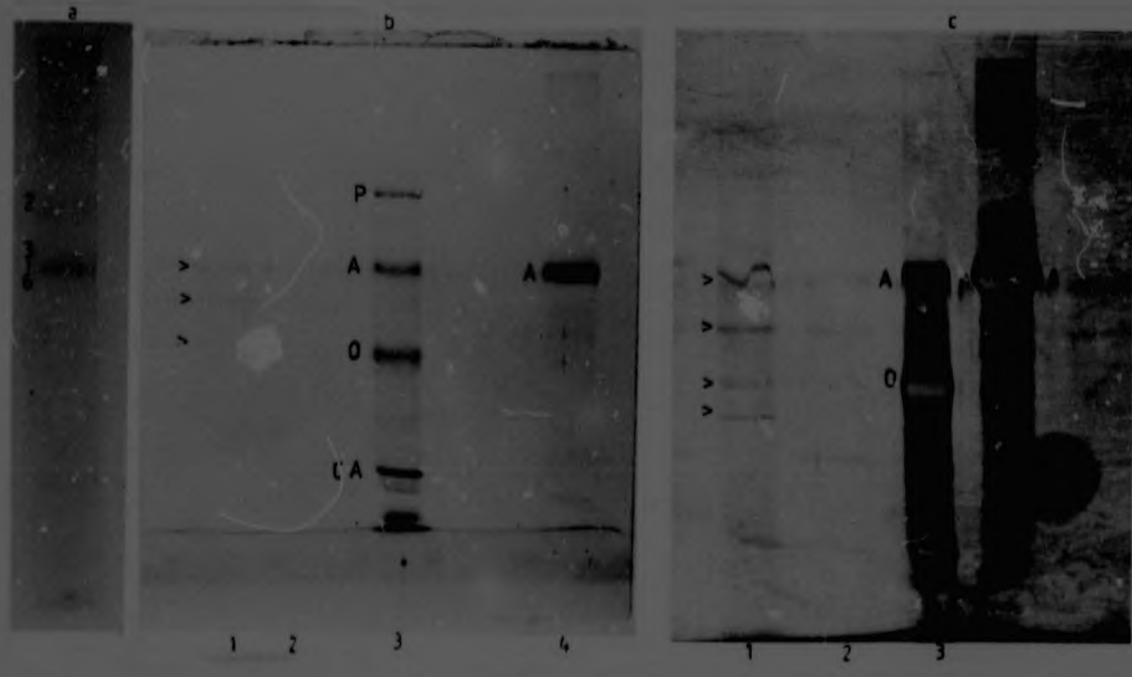
Fig.5:5. Kinetics of cPDE activity of two forms of the enzyme in human leiomyoma of the uterus (cAMP tested).
Enzyme activity was derived from extracted bands 1 and 2 from native polyacrylamide-gel electrophoresis and was assayed at cAMP concentrations between 0.5 uM and 100 uM. Results (a) of a single representative experiment, drawn as an Eadie-Hofstee plot, are shown for band 1. The two kinetically distinct forms of band 2 (b) are presented as an Eadie-Hofstee plot after reconstruction of the raw data according to Spears *et al.* (1971).



Partial purification of the major form of cPDE in cytosols of human leiomyoma.

The specific activity of the high affinity component of the crude leiomyoma supernatant was 0.036 nmol/min/mg protein. Fractionation of the cytosol on DE-52 anion exchange resin between 0.1-0.4 M NaCl followed by theophylline-Sepharose and sucrose gradient ultracentrifugation increased the specific activity to 2.68 nmol/min/mg protein that is a 74 fold purification. It is impossible to calculate an accurate recovery for bands 3-6 because of the presence of the other forms in the crude leiomyoma cytosol. Purification through the same steps described above except that the sucrose gradient centrifugation was replaced by preparative electrophoresis (Fig. 5:6(a)) as described in materials and methods produced enzyme which on SDS gel-electrophoresis yielded the pattern of four bands at Mr 65000 ± 3000, 50000 ± 2000, 44000 ± 2000, 41000 ± 2000 (Fig. 5:6(b) and (c)). The protein standards for SDS electrophoresis were phosphorylase b (P) 94000, albumin (A) 67000, ovalbumin (O) 43000 and carbonic anhydrase (CA) 30000. The molecular masses of the subunits were determined from the method of Weber and Osborn (1969).

Fig. 5:6. (a) Sample of affinity purified fraction of leiomyoma (0.1-0.4 M NaCl eluate from DE-52) was run at 7.5 % gel concentration for cPDE activity depicting band 2 and cluster of bands 3-6. The (b) figure shows the subunits (Coomassie 3DS) of the purified cPDE of leiomyoma in lane 1, a blank with buffer in lane 2 and protein standards in lane 3 (phosphorylase b P, albumin A, ovalbumin O carbonic anhydrase CA) and albumin in lane 4. The (c) figure depicts the same as (b) but the more sensitive silver stain was applied.



Discussion

The initial part of this discussion will compare the present results with purified human and mammalian cPDE enzymes described in other tissues and cells. The second part will summarize the findings on either crude or partially pure preparations from human and mammalian uterine tissues.

Human leiomyoma soluble cPDE activity could be resolved into seven bands of enzyme activity by electrophoresis at lower gel concentrations. This was observed for both cAMP and cGMP at a substrate concentration of 100 uM. Indeed, this pattern, except for band 7, was characteristic in over five separate preparations of this tissue. Homogenizing the tumour in buffers A, B, or C also resulted in the observed pattern of activity for either cAMP or cGMP (Fig. 5:1b). The measured relative mobilities (Rf) for cAMP or cGMP were indistinguishable. Invariably, band 5 exhibited the majority of the staining activity for both cAMP and cGMP. Caution in the interpretation of this observation is necessary since it has been reported that lead ions in the incubation medium inhibit some forms of cPDE more than others (Nemoz *et al.* 1983). I did utilize half the concentration of lead ion, 1.5 mM instead of 3 mM, in an attempt to reduce the possibility of this effect. In addition, elution of cPDE from unstained gel slices, showed the highest activity to be associated with band 5 for both low and high Km forms (1 uM and 100 uM cAMP)(for results at 1 uM cAMP see Table 5:3). Multiple forms, about 5, of cPDE have been described in

rat brain using the technique of isoelectricfocusing on granular gel plates (Nemoz *et al.* 1985).

Ferguson plot analysis of the leiomyoma activity bands revealed four different molecular mass species and a cluster of four charge forms in the group of bands 3-6. Evidence that the Band 1 form was a distinct enzyme might be summarized as follows: 1) This form was eluted at 0.1 M ionic strength from DE-52 cellulose 2) The other 0.1-0.4 M ionic fraction, which contained bands 2-7, when further purified on theophylline-Sepharose did not show interconversion to Band 1 when analyzed on native polyacrylamide-gel electrophoresis (Fig. 5:6(a)). 3) Kinetic analysis of the form indicated linear Michaelis-Menten kinetics from Eadie-Hofstee plots and a higher affinity for cAMP compared with band 2. 4) The form was inhibited by preincubation with EGTA (1 mM). 5) Band 1 exhibited the highest molecular mass of apparent Mr of 229000 ± 4000 and Yo = 2.49 but this higher value was insufficient to be explained as simple aggregation of the lower molecular mass isoenzymes 3-6 which were also EGTA inhibited. There have been reports in the literature, based on different tissues and species, of calcium/calmodulin dependent forms, that is type I cPDE enzymes, hydrolyzing both nucleotides with similar native molecular masses of about 240000 (Hidaka and Asano 1976; Tucker *et al.* 1981) to the band 1 form. The reported Mr of around 200000 for cGMP-stimulated phosphodiesterases in calf liver (Yamomoto *et al.* 1984(a)) (type II cPDE) and 240000 for bovine adrenal and heart tissues (Martins *et al.* 1982)(type II cPDE)

are also not dissimilar to that of the band 1 form. I am, however, unable to comment on the effect of cGMP on the hydrolysis of cAMP by the various enzyme forms in leiomyoma since this was not examined.

Band 2 appeared to be a distinct form of cPDE particularly in the characteristic of being totally impervious to preincubation with EGTA (Fig. 5:1(c) and (d)). It could therefore be regarded as calcium independent and hence calcium/calmodulin independent. Furthermore, the extracted enzyme from non-denaturing polyacrylamide electrophoresis gels did not show increased activity in the presence of exogenous calcium and calmodulin. The anomalous kinetics with approximate Km values for band 2 of 5.8 uM and 37 uM are not dissimilar from those reported for the enzyme(s) in crude cytosols of rat uterus of 3 uM and 20 uM (Strada *et al.* 1981) or values of 2 and 36 uM found for soluble uterine phosphodiesterase from diethylstilbestrol-treated immature rats (d'Auriac and Meyer 1973). The apparent molecular mass reported by Strada and associates (1981) was lower, that is 7.0 S (ca. 130000-140000) compared to my calculated Mr of 186000 $\pm$ 4000 for band 2. An earlier report by Gardner and co-workers (1978) on rat uterus yielded a higher value for the Sedimentation Coefficient of the enzyme namely 7.9 S.

Bands 3 to 6 were characterized as charge species of apparent Mr 174000 $\pm$ 4000. These forms were all largely inhibited by preincubation with 2 mM EGTA, although lower concentrations, <1 mM, did not inhibit forms 3 and 4 very strongly (Fig. 5:1(c)).

This would imply these forms are calcium/calmodulin dependent (Ho *et al.* 1977). Anomalous forms of different Rf were observed in the cytosol incubated with EGTA (Fig. 5:1(c) and (d)). Clearly more detailed research on these species is required. Since it was the bands 3-6 which were partially purified and shown to consist of subunits of apparent Mr 65000 ± 2000, 59000 ± 3000, 45000 ± 2000 and 41000 ± 2000 on SDS electrophoresis, certain inferences may be drawn. I was unable to detect the presence of calmodulin in the purified fraction when subjected to SDS electrophoresis under the conditions used. This would suggest that calmodulin was either lost during the earlier stages of the purification procedure or was tightly bound to the cPDE subunits on the SDS gels, which is unlikely.

The overall purification of the leiomyoma enzyme was only 74 fold because heavy losses in enzyme activity were incurred. This was probably due to the relatively low starting protein concentration (35 g/run) and the very labile nature of the isoenzymes. The two lower molecular mass subunits might be the proteolytic fragments described by Tucker and co-workers (Tucker *et al.* 1981) for the type I(a) cPDE enzyme even though PMSF was present in all solutions. The structure of the native soluble human leiomyoma isoenzymes could theoretically be composed of two subunits of type I cPDE and two calmodulin molecules A_2C_2 (ca. a mass of 160000)(La Porte *et al.* 1979; Sharma *et al.* 1984). I do not consider these forms to be similar to the 175000 form (type IV cAMP PDE) isolated in human platelets (Umekawa *et al.* 1984; Grant and Colman, 1984) as

their reported Km was much lower, that is, <0.4 uM compared to my range of 17-24 uM. In addition, the leiomyoma forms appeared to be equally active when gels were incubated in the presence of either cAMP or cGMP and they appeared to be calcium/calmodulin dependent because of their sensitivity to EGTA (see Chapter 1 for description of type IV cAMP PDE). The cAMP PDE (type II cPDE) isolated in calf liver with Mr of 174000 by Yamomoto and co-workers (1984(a)) more closely resembles the leiomyoma enzyme also exhibiting non-linear kinetics with an apparent low Km component of approximately 2 uM. The authors did not report the Km of the low affinity component. Nonetheless, the leiomyoma isoenzymes appear to be calcium/calmodulin dependent which is not the case with type II cPDE enzymes. It is interesting to speculate that three of the forms of the bands 3-6 could represent the two homodimers and one heterodimer described in bovine brain of a calmodulin-dependent cPDE (type I(a) cPDE (Sharma *et al.* 1984; Hansen and Beavo 1986). The activity form, represented by band 7, was not always present, particularly in the leiomyoma, and might represent a proteolytic fragment which nonetheless was inhibited by EGTA, unlike the fragments described by Tucker and associates (1981) which are not.

The latter part of this discussion centres on research related to uterine cPDE forms and the findings of five different groups will be summarized.

The first group, reported once on the levels of cPDE and adenyl cyclase in rat uterus during the oestrus cycle (Sim and

Chantharaksri 1974). These authors reported that in crude homogenates both enzyme activities were highest at met-oestrus but lowest at pro-oestrus suggesting steroid hormone control of the two enzymes.

The second group, based their studies on cPDE in crude supernatants of rat uterus, where there appeared to be at least two different kinetic forms (only cAMP hydrolysis was measured) both of which were inhibited by ATP (d'Auriac and Meyer 1973). It also appeared that, angiotensin II at levels that could cause contractions of rat uteri, had no effect on the soluble cPDE activities.

A third group have extensively studied cPDE, using both cAMP and cGMP, in monkey uterine tissues at different stages of development (Beatty *et al.* 1979). Beatty and co-workers have reported that the majority of the cPDE activity is associated with the supernatant (about 80%) in agreement with the findings by Bar (1974) in the rat myometrium supernatant and Leroy and associates (1985) in supernatants from human pregnant myometrium. They have also shown that cAMP metabolism in myometrium was altered during the menstrual cycle (Beatty et al. 1977) suggesting regulation of cAMP levels to be under steroid hormone control. The Beatty team published results of non-linear kinetics for cAMP hydrolysis in monkey myometrium but did not characterize the cPDE further or report Km or Vmax values (Beatty *et al.* 1976). This latter team did however provide further characterization of cPDE in their later comparative study of spayed macaque myometrium versus pregnant

myometrium (Beatty *et al.* 1979). The enzyme in crude cytosols of pregnant and spayed myometrium gave anomalous double-reciprocal plots of apparent Km values for cAMP of 4.6 to 18.7 uM and 2.9 to 24.9 uM respectively. The Vmax values for both tissues were very similar and were about 6 nmol/min/mg protein. My Vmax values for the separated soluble cPDE forms of human leiomyoma (Table 5:3) were all considerably higher and ranged from 12 to 125 nmol/mg protein/min. Linear kinetics for cGMP hydrolysis was observed for the enzymes in soluble pregnant myometrium (Km=7.6 uM, Vmax=9.3 nmol/min/mg protein) but not always for the enzyme in spayed monkeys, suggesting more than one form of the enzyme, as would be expected in crude cytosols. Furthermore, cGMP at low concentrations, could stimulate cAMP hydrolysis which is a feature of the type II cPDE form. Finally, the group concluded that levels of cPDE were raised in pregnant myometrium compared to spayed myometrium expressed on the basis of DNA content but the situation was reversed when comparisons were based on either wet tissue mass or nitrogen (protein) content (Beatty *et al.* 1979).

The fourth team have based their studies on supernatants from homogenates of rat uterine tissues and first suggested that in this tissue cAMP PDE (cGMP hydrolysis was not measured) was under the control of steroid hormones (Stancel *et al.* 1975). Their subsequent studies on partial purification of rat uterus cPDE using DEAE-cellulose ion-exchange chromatography followed by sucrose gradient ultracentrifugation consistently revealed a

single form of cPDE of about 7.9 S, as evaluated by sedimentation in the ultracentrifuge, which could hydrolyze both cAMP and cGMP (Gardner *et al.* 1978). This form, however, is in contrast to the report previously mentioned on crude supernatants of rat uterus (d'Auriac and Meyer 1973). A further study by the fourth group, based on limited proteolysis studies of the enzyme using trypsin, indicated the catalytic sites for cGMP and cAMP to be separate (Strada *et al.* 1981). Of particular importance was their finding that limited proteolysis of one of the DEAE-cellulose eluted forms resulted in two forms when rerun on DEAE-cellulose. This report by Strada and colleagues (1981) was disputed by Keravis and colleagues (1981) who reported non-linear kinetics for cAMP of Km 3 to 20 uM and linear kinetics for cGMP with a Km of about 3 uM. These Km values are not dissimilar to those reported by d'Auriac and Meyer (1973)(2 and 36 uM for cAMP) or to those reported by Beatty and associates (1979) or indeed to my own findings. The fourth group did not show stimulation of cAMP hydrolysis by low concentrations of cGMP (see Beatty *et al.* 1979).

The final group have studied cPDE in either human myometrium, human placenta or cultured myometrial cells (Ferre *et al.* 1975; Ferre *et al.* 1977; Vallet-Strouve *et al.* 1984; Leroy *et al.* 1985). At least two cPDE activities were reported for homogenates of human placenta and imidazole was demonstrated to stimulate the lower affinity form (Km = 1.36 mM)(Ferre *et al.* 1975). The high affinity cAMP PDE (cGMP hydrolysis was not

measured), exhibited a Km of 10 uM and Vmax of 0.63 nmol/min/mg protein, and was weakly inhibited by prostaglandins but strongly inhibited by progesterone. They repeated these experiments in cytosols of human pregnant myometrium and, from the crude homogenates, two forms of cAMP PDE were isolated (higher affinity form with Km of 12.6 uM, Vmax of 0.5 nmol/min/mg protein; lower affinity form Km of 90 uM, Vmax of 1.0 nmol/min/mg protein). The stimulatory effect of imidazole for the activity of the lower affinity enzyme was apparent and, as for placenta, indomethacin and progesterone (non physiological concentrations *in vitro*) were found to inhibit the higher affinity form. Of interest, oestradiol 17B and several oestradiol derivatives were shown to have negligible inhibition on cPDE *in vitro* (Ferre *et al.* 1977). Apparently, progesterone has been shown both *in vivo* and *in vitro* to inhibit human uterine contraction (Ferre *et al.* 1977). Subsequent work by this group on myometrial cells in culture, have shown that physiological concentrations of either progesterone or oestradiol inhibited cAMP PDE activity (cGMP hydrolysis was not measured) (Vallet-Strouve *et al.* 1984). This effect has also been reported by others (Stancel *et al.* 1975; Beatty *et al.* 1979). Furthermore, *in vivo* administration of oestrogen decreased cPDE activity in rat uterus (Gardner *et al.* 1978) whereas only progesterone decreased PDE activity in macaque myometrium (Beatty 1978). Recently, a report on murine oocyte maturation has demonstrated that either progesterone or Beta-oestradiol or testosterone inhibited cPDE activity in

oocyte extracts (Kaji *et al.* 1987). Clearly there appear to be some conflicting results regarding the inhibitory effects by progesterone and oestradiol on the cPDE activity. It is possible that the discrepant observations might reflect differences in the oestradiol to progesterone ratio which varies considerably either during the menstrual cycle, or stages of pregnancy or as a result of species differences. Also, discrete forms of cPDE exhibiting differential inhibition by the two hormones might be synthesized either during development, the menstrual cycle, pregnancy or as a result of species differences.

The reported kinetics for cAMP hydrolysis in supernatants of human myometrial cells in culture were non-linear in agreement with the other groups (Stancel *et al.* 1975; Beatty *et al.* 1976; Ferre *et al.* 1977; Beatty *et al.*1977). The apparent Km values of the soluble cPDE in myometrial cells for cAMP were 0.8 uM and 40 uM with respective Vmax values of 0.06 and 1.4 nmoles/min/mg protein. The latter values increased by 100% following several successive subcultures. Futhermore, a decrease in cAMP PDE activity was noted with high densities of cells and *vice versa* (Vallet-Strouve *et al.* 1984). The most recent report by this group has been based on longitudinal sections of human myometrium at the end of pregnancy (Leroy *et al.* 1985). It appears that less than 10% of either cAMP or cGMP PDE activity was associated with the particulate fraction. Two soluble cPDE forms were isolated using DEAE-cellulose chromatography and one form (peak I) most closely resembled the

type I cPDE and the second eluting form (peak II) appeared to be similar to the type IV cAMP PDE. I did not detect the type IV cPDE in the cytosols of human nonpregnant myometrium using my gel method coupled to the cPDE activity stain. That is, I observed the same cPDE activity profile for human myometrium cytosol in the presence of either cAMP or cGMP.

Sucrose gradient ultracentrifugation of the soluble fraction of "end of pregnancy" myometrium (Leroy *et al.* 1985) gave rise to three peaks A,B and C of which, C, appeared to be type IV cAMP PDE. The peaks A and B (hydrolyzing both substrates) could also be resolved on sucrose gradients when only peak I was loaded suggesting at least three distinct forms of cPDE in human pregnant myometrium. Both substrates exhibited non-linear kinetics with apparent Km values for cAMP in crude supernatant of 2.8 uM and 57 uM ; for cGMP of 0.83 uM and 12 uM. The peaks I and II obtained on DEAE chromatography revealed different kinetic properties: Peak I (type I cPDE) exhibited Km values for cAMP of 28 uM and 4200 uM with respective Vmax values of 0.4 and 2.0 nmol/min/mg protein; Km values for cGMP of 1.7 uM and 16 uM with respective Vmax values of 0.04 and 0.22 nmol/min/mg protein; Peak II (type IV cAMP PDE) gave Km values for cAMP of 10 uM and 1000 uM with respective Vmax values of 0.6 and 4.1 nmol/min/mg protein. The estimated molecular masses of A, B and C from sucrose gradient studies suggested 110000 daltons, 47000 daltons and 18000 daltons respectively. It appears that, in agreement with Leroy and associates (1985), in human leiomyoma versus "end of pregnancy" myometrium there are

at least three distinct forms of cPDE and that there is more than one form of type I cPDE. My results, however for cAMP hydrolysis by band 1 form (type I cPDE) and bands 3-6 (type I cPDE) yielded much higher affinities for the substrate and also higher Vmax values in comparison to Leroy and worker's (1985) partially purified "peak I". In fact my values do not differ much from their results reported for crude supernatants of human pregnant myometrium. However, the molecular mass values are radically different. Whether these discrepancies reflect true differences between cPDE forms in human leiomyoma cytosols versus human pregnant myometrium cytosols or are the result of differences in methodology remains to be resolved. It is worth noting, though, based on my own experiences with sucrose gradient ultracentrifugation, that if leiomyoma supernatants, either crude or partially pure, were frozen and rethawed or stored for more than three weeks at -70° C, lower molecular mass forms of cPDE were detected and were regarded as proteolytic but active cPDE enzyme fragments.

Further observations, based on the activity pattern in non-denaturing polyacrylamide electrophoresis of human leiomyoma cyclic nucleotide phosphodiesterase revealed that the cPDE enzyme pattern did not differ for either human myometrium or normal uterus (composed of both myometrium and endometrium). In addition, the myometrium tissue displayed denser staining in all activity bands at the same soluble protein concentrations compared to that of leiomyoma from the same patient (Figs. 5:1a, 5:1b, 5:1c, 5:2b and 5:2c) indicating higher cPDE

activity in myometrium tissue. This finding was most interesting in that when I studied the oestrogen and progesterone receptor content of the two tissues both receptor types were significantly higher in leiomyoma ($p = 0.0002$) (Sadan *et al.* 1987). Therefore, there appears to be an inverse correlation between oestrogen/progesterone receptors and the level of cPDE activity which is in agreement with the findings of Etingof *et al.*(1984) who studied oestrogen receptors and cPDE levels in both human and uterine tissues.

Of interest, a recent report on the kinetic characteristics of cPDE in cruda cytosols of human pregnant and nonpregnant myometrium has shown a markedly lower Vmax in the pregnant tissue (Kofinas *et al.* 1987). This suggests steroid hormonal influences on the soluble cPDE activity in human myometrium during pregnancy. Nonetheless, in view of the multiple forms of cPDE that I found in human myometrium cytosols, studies of cPDE kinetics based on crude myometrium cytosols are difficult to interpret.

Characterization of soluble uterine cyclic nucleotide phosphodiesterases in rat uterus has only revealed one form with anomalous kinetics (Gardner *et al.* 1978; Strada *et al.* 1981). Several recent reports in the literature indicate that there are distinct species differences in the four main types of soluble cyclic nucleotide phosphodiesterases, referred to previously, in either one or more of the following: kinetic parameters, physical properties or regulation by pharmacological agents (Geremia *et al.* 1984; Mullaney and

Clegg, 1984; Mutus *et al.* 1985; Pyne *et al.* 1986).

The diverse molecular mass species and charge forms in the human uterine tissues that I studied might also be ascribed to: 1) the release of membrane-bound forms due to the extremely low temperature of liquid nitrogen (-196° C) (not previously used to my knowledge) and pulverizing of the tissue in this medium. 2) The result of endogenous proteolysis during processing. The very consistent activity pattern observed in all the tissue cytosols would tend to refute this but I cannot discount it entirely. It was found that repeated freezing and thawing of the cytosols considerably lowered the intensity of staining and resulted in poorly resolved bands. In addition, more anionic bands were sometimes observed.

In conclusion, I consider that human leiomyoma possess three distinct soluble cPDE forms and that there also exist isoenzymes. The human myometrium and uterus also contain these three forms. The method of slab gel electrophoresis described here may prove useful in the analysis of cPDE enzymes in tissue available in only small quantities, as is the case in man. It should also be of potential value in the assessment of inhibitor sensitivities in different tissues.

CHAPTER SIX

CONCLUSIONS

Since most of the work in this thesis has been discussed in separate sections, it would be pertinent to consolidate the information in order to gain a clearer perspective of the soluble cPDE enzymes in mammalian tissues and the possible clinical significance of my findings. A brief overview of cPDE forms in more primitive organisms, where some gene sequencing has been achieved, will be summarized prior to my findings of the soluble cPDE enzymes in mammalian tissues.

The cPDE enzymes of primitive eukaryotes.

In view of the overwhelming evidence that cPDE enzymes hold key positions in cellular biochemical regulation and the fact that they are the only specific catabolic enzymes for the ubiquitous substrates, cAMP and cGMP, I would tend to expect the cPDE enzymes to be highly conserved between species. In other words, perhaps only very few mutations would be advantageous and, most in fact, deleterious to the vital function of these regulatory enzymes. Thus reported findings of cPDE enzymes in more primitive organisms ought to answer some of the questions that have arisen from *in vitro* studies based on cPDE enzymes in

mammalian tissues. Certainly, *in vitro* studies of cPDE enzymes in eukaryotes have demonstrated multiple forms of cPDE. In my opinion, however, a disconcerting problem has been the fact that purified cPDE enzymes of the same classification can differ considerably : th respect to kinetic parameters and physical characteristics between mammalian species. A case in point would be the purified soluble type II cPDE in bovine liver (Martins *et al.* 1982) compared to the soluble purified type II cPDE in rat liver (Pyne *et al.* 1986) which represents just one of many dicrepancies (see Introduction). In brief, are the multiple cPDE forms observed *in vitro* between species either the result of: different structural genes; the same gene but considerably variant as a result of accummulated mutations; differential processing of the mRNA's in the nucleus or cytoplasm; differential covalent modification of the translation products; or artifacts arising from different *in vitro* separation techniques? Furthermore, the multiple forms observed within different tissues within the same species could be either: different structural genes that may be differentially expressed or suppressed in specific tissue types; one structural gene that is differentially processed in certain tissues; or artifacts that arise from variations of *in vitro* methodologies.

Recently, a partial or complete DNA sequencing has been attained in *Drosophila melanogaster* (Davis and Kauvar; Chen *et al.* 1986), in yeasts (Sass *et al.* 1986; Nikawa *et al.* 1986) and slime mold (Lacombe *et al.* 1986). It appears that there are two

genetically distinct forms of cPDE in *Drosophila* (Walker and Kiger 1984; Chen *et al.* 1986). The form I enzyme resembles the mammalian type I cPDE in mass but not kinetic parameters and is regulated by a calmodulin-like protein. The form II cPDE, which is associated with the dunce gene mutation, most closely resembles the mammalian type IV cPDE. In the yeast, *Saccharomyces cervisiae*, two genes, PDE 1 and PDE 2, have been cloned and encoded respectively by Nikawa *et al.* ( ) and Sass *et al.* (1986). An interesting study, based on comparisons of partial amino acid sequences between type I cPDE from mammalian brain, type II cPDE from bovine heart with those protein sequences (derived from the nucleotide sequences of the genes) of the yeast PDE 2 gene and the *Drosophila* form II gene, has demonstrated a conserved domain in all four of the cPDE enzymes from these widely differing species (Charbonneau *et al.* 1986). The yeast PDE 1 gene, however, showed no apparent homology with the above four cPDE enzymes but did show homology with the *Dictyostelium discoideum* extracellular cPDE enzyme (Nikawa *et al.* 1987). The latter finding suggests that the slime mold cPDE might represent a structural gene for cPDE not found in higher organisms. It should be noted that the above cPDE genes which have been sequenced in the more primitive eukaryotes might not be the only genes present and it is quite possible that new cPDE genes remain to be identified.

In summary, therefore, there appears to be conservation of amino acid sequences in some of the cPDE enzymes classified as type I, type II and type IV (Charbonneau *et al.* 1986). Evidence

presented by the Charbonneau team (1986) has implied that, since the homology between mammalian type I cPDE and and type II cPDE was rather limited, the respective structural genes were probably discrete. The use of polyclonal antibodies by Sarada and associates (1982) has also demonstrated shared homology between mammalian type I cPDE enzymes with those of mammalian type IV cPDE. One exception to the above was a report by a group (Shenolikar *et al.* 1985) in which they were unable to demonstrate any cross reactivity, using Western blot analysis, between soluble bovine brain calmodulin-stimulated cGMP PDE (type I (c) cPDE, see Introduction) and soluble type IV cPDE of dog kidney. The type III (a) and (b) cPDE enzymes are uniquely associated with the lens of the eye and there is strong evidence, derived from two dimensional tryptic peptide mapping (Takemoto *et al.* 1984), for interspecies conservation of the type III (a) cPDE. Furthermore, Barbehenn and associates (1985) have demonstrated antigenic similarity between type III (a) and type III (b) enzymes. Clearly, evidence thus far, seems to favour the theory that the four main cPDE classifications, type I, type II, type III and type IV cPDE represent discrete structural genes, some of which share a degree of sequence homology.

The findings outlined above, however, have still not satisfactorily resolved the reported differences in properties within a cPDE type either between species or within different tissues of the same organism. It is indeed likely that many of these differences can be attributed to artifacts arising from

in vitro methodology rather than to true physical variations in the cPDE forms. Some of the major problems, not the least of which are the facts that the cPDE enzymes are both highly active and present in small amounts, include: the sensitivity of cPDE enzymes to either endogenous or exogenous proteolysis and the ability of fragments to remain catalytically active, such as the irreversible activation of type I cPDE by proteolysis with concomitant loss of responsiveness to calcium/calmodulin (review, Krinks *et al.* 1984); either similar or differential sensitivity of cPDE enzymes to an immense array of agents ranging from endogenous cations, sulphydryl compounds, small peptides, phospholipids and hormones to exogenous effectors such as imidazole and many exogenous inhibitors (see Introduction); the effect of pH which irreversibly inactivates cPDE under acidic conditions (pH < 5) (Strada *et al.* 1978); hydrophobic interactions (Van Inwegen *et al.* 1976); and temperature sensitivity (Strada *et al.* 1978).

The above difficulties explain, in large part, why so few studies of the cPDE enzymes in human and murine tissues have been published. Since the study of these enzymes in human and murine tissues is only possible with small tissue specimens, it was necessary to adapt the method of non-denaturing polyacrylamide gel electrophoresis coupled to the specific cPDE activity stain. This technique can enable as little as 0.5 mg of soluble protein to effect a separation of the soluble cPDE enzymes.

Comparative findings of the cPDE forms in murine and human tissue cytosols.

My studies, which have dealt mainly with the characterization and partial purification of the soluble cPDE enzymes in murine tissue cytosols and human mammary and uterine cytosols, consistently demonstrated a significant portion of the total soluble cPDE activity to be calcium/calmodulin dependent. This was proved by inhibition to the following; EDTA, a calcium chelator; EGTA, also a calcium ion chelator; and the specific inhibitor, trifluoroperazine. Furthermore, when these cPDE activity forms were excised and eluted from gels, the enzymes were not further activated in the presence of exogenous calcium/calmodulin which suggested that nd PAGE did not separate calmodulin from the type I cPDE forms. Of those cPDE activity forms that were shown to be calmodulin/dependent (type I cPDE), there appeared to be activity bands that were common to all the tissue cytosols I studied. In the murine tissue cytosols, the Rf range of a common calcium/calmodulin sensitive cPDE activity band was 0.28-0.35 at 7.5% gel concentration (Table 2:4, repeated below). In human mammary tumour cytosols (Fig. 4:1a (c)) the EGTA-sensitive cPDE activity bands, 2 to 4, exhibited an Rf range of 0.28-0.34 at 7.5% gel concentration which is within the same range as that found for the common calmodulin/dependent cPDE activity band in all the murine tissue cytosols. Similarly, the normal human mammary cytosol bands 2 and 3 had an Rf range of 0.31-0.34 at 7.5% gel

concentration. Lastly, the human uterine cytosol bands 3 to 5, also EGTA-sensitive (Fig. 5:1(a)), exhibited an Rf range of 0.29-0.35 (results summarized below in Tables 2:4, 6:1 and 6:2). The two faster migrating cPDE bands observed only in murine mammary tumour cytosol (5 to 6, Rf range 0.34-0.41 and EDTA-sensitive) were, however, apparent in all the human cytosols, except for the fastest migrating cPDE activity form in normal human mammary cytosol, and are summarized: human mammary tumour cytosol (bands 5 to 4, Rf range 0.35-0.40); normal human mammary cytosol (band 4, Rf range 0.35-0.36); uterus, myometrium and leiomyoma cytosols (bands 6 to 7, Rf range 0.36-0.41) (see Tables 2:4, 6:1 and 6:2.)

Unfortunately, I am unable to comment on the comparative molecular masses of the soluble cPDE forms, calculated by Ferguson plot, which were found to be higher for all the murine tissue cytosols. The reason for this is that I used protein standards that were optimized for running on nd PAGE at the start of the human tissue soluble cPDE studies and the new standards appeared to yield lower molecular masses. Nonetheless, my findings did reveal that the faster migrating cPDE activities appeared to be type I cPDE isoenzymes based on the facts that they were either inhibited by EDTA or EGTA and also the forms exhibited nearly identical molecular masses but possesed discrete charge densities (Yo) within the different tissue cytosols.

Another soluble cPDE activity band that was consistently demonstrated, using non-denaturing polyacrylamide gel

electrophoresis coupled to the cPDE activity stain, with the exception of murine brain cytosol, was the EDTA/EGTA-insensitive cPDE enzyme. When this cPDE activity form was excised and eluted from gels, the cPDE activity was not increased in the presence of exogenous calcium/calmodulin and I considered this cPDE activity to be calcium/calmodulin independent. In murine tissue cytosols, except for murine brain and the EDTA-insensitive band 1 of murine heart, the EDTA-insensitive cPDE activity bands in murine cytosols had an Rf range of 0.24-0.28. This same characteristic cPDE activity band had an Rf range of 0.25-0.27 in human mammary cytosols and an Rf range of 0.26-0.27 in human uterine cytosols (see Tables 2:4, 6:1 and 6:2 below). Kinetic analysis of this EGTA-insensitive form from human mammary tumour cytosols and human leiomyoma cytosols was achieved by eluting the band of interest (note: not exposed to lead ions) from polyacrylamide gels and then performing the cPDE radioassay. The kinetic parameters of the calcium/calmodulin independent cPDE enzyme in human mammary tumour cytosol (for cAMP, Km values of 3.8 uM and 50 uM with respective Vmax values of 4.5 and 25 nmol/mg protein/min) were not dissimilar from those reported for the same enzyme in human leiomyoma cytosol (for cAMP, Km values of 5.3 and 37 uM with respective Vmax values of 12 and 64 nmol/mg protein/min). In both tissues, the cPDE enzyme exhibited complex kinetics with apparent negative cooperativity. This suggests that the EGTA-insensitive cPDE activity band is either indicative of more than one cPDE enzyme, possibly not fully

separated on nd PAGE, or that the enzyme is allosteric with different catalytic binding sites.

The calcium/calmodulin cPDE activity band with the characteristic Rf range of 0.24-0.28 (molecular mass range of 168000 to 186000 daltons in human cytosols) which I observed in all the tissue cytosols, except murine brain, could be regarded as similar to either the soluble type II cPDE described in bovine heart (Martins *et al.* 1982) or calf liver (Yamomoto *et al.* 1983(a)) or a soluble type IV cPDE purified from human platelets (Umekawa *et al.* 1984). The soluble type II cPDE from heart has been reported to have a molecular mass of 240000 daltons and Km for cAMP of 30 uM (Vmax not given) (Martins *et al.* 1982). The similar enzyme in calf was demonstrated to have a mass of 201000 daltons, Km for cAMP of about 30 uM and a Vmax of 170 umol/mg protein/min (Yamomoto *et al.* 1983(a)). The soluble type IV cPDE has been shown to have a mass of 175000 daltons, Km of 0.34 uM (with 0.11 uM for cGMP) and a Vmax of 85 nmol/mg protein/min (Umekawa *et al.* 1984). The very high Vmax (one thousand fold higher, in umol as averse to nmol/mg protein/min) reported for the calf liver cPDE would seem to suggest a closer resemblance of my form to the unusual soluble type IV cPDE. The soluble type IV enzyme in human platelets is unusual in that it has been demonstrated to exhibit an almost equal hydrolysis rate for both cAMP and cGMP (Umekawa *et al.* 1984) whereas the type IV cPDE enzymes tend to be cAMP-specific (see Introduction section on type IV cPDE's). Thus the dichotomy still remains as to why the soluble cPDE types vary

so considerably with respect to both physical and kinetic parameters in different species. Only further biochemical studies, using several different separation techniques, will possibly resolve this question.

Table 2:4. Relative mobility (Rf range) of the cPDE activity bands of murine tissue cytosols observed in 7.5% nd PAGE coupled to the cPDE activity stain as shown in Fig. 2:4a for both cAMP and cGMP. The results are the mean of four experiments.

	Rf range from top of gel	Tissue and band position (Fig. 2:4)
A.	0.095-0.11	kidney (1)
B.	0.12 -0.14	heart (1)
C.	0.24 -0.28	tumour (1), heart (2), liver (1), kidney (2),
D.	0.29 -0.35	bovine heart forms, heart (3), brain (1), liver(2), kidney (3), tumour (2)
E.	0.34 -0.36	tumour (3)
F.	0.37 -0.41	tumour (4)

Table 6:1. Relative mobilities (Rf range) of the cPDE activity bands of human mammary cytosols observed in 7.5 % nd PAGE coupled to the cPDE activity stain as shown in Fig. 4:1a (a). The calculated Rf values were the same for either cAMP or cGMP. The results are the mean of three experiments.

	Rf range from top of gel	Tissue and band position (Fig. 4:1a (a))
A.	0.25-0.27	normal breast(1), breast tumour (1)
B.	0.29-0.30	breast tumour (2)
C.	0.31-0.32	normal breast (2), breast tumour (3)
D.	0.33-0.34	normal breast (3), breast tumour (4)
E.	0.35-0.36	normal breast (4), breast tumour (5)
F.	0.39-0.40	breast tumour (6)

T.ble 6:1. Relative mobilities (Rf range) of the cPDE activity bands of human mammary cytosols observed in 7.5 % nd PAGE coupled to the cPDE activity stain as shown in Fig. 4:1a (a). The calculated Rf values were the same for either cAMP or cGMP. The results are the mean of three experiments.

	Rf range from top of gel	Tissue and band position (Fig. 4:1a (a))
A.	0.25-0.27	normal breast(1), breast tumour (1)
B.	0.29-0.30	breast tumour (2)
C.	0.31-0.32	normal breast (2), breast tumour (3)
D.	0.33-0.34	normal breast (3), breast tumour (4)
E.	0.35-0.36	normal breast (4), breast tumour (5)
F.	0.39-0.40	breast tumour (6)

Table 6:2. Relative mobility (Rf range) of the cPDE activity bands of human uterine cytosols observed in 7.5% nd PAGE coupled to the cPDE activity stain. The calculated Rf values were the same for either cAMP or CGMP. The results represent the mean of four experiments.

	Rf range from top of gel	Tissue and band position (Fig. 5:1(a))
A.	0.18-0.19	uterus (1), myometrium (1), leiomyoma (1)
B.	0.26-0.27	uterus (2), myometrium (2), leiomyoma (2)
C.	0.29-0.30	uterus (3), myometrium (3), leiomyoma (3)
D.	0.31-0.32	uterus (4), myometrium (4), leiomyoma (4)
E.	0.33-0.35	uterus (5), myometrium (5), leiomyoma (5)
F.	0.36-0.38	uterus (6), myometrium (6), leiomyoma (6)
G.	0.40-0.41	uterus (7), myometrium (7), leiomyoma (7)

From the above Tables it can be seen that the murine kidney cytosol band 1 cPDE form, murine heart band 1 and the human uterine band 1 represent unique cPDE forms with their own characteristic relative mobility under controlled conditions. It is possible that the murine kidney band 1 cPDE could represent an aggregate of murine kidney band 3 (both EGTA-sensitive) where the mass of band 1 was 395000 daltons and band 3 was 245000 daltons (Table 2:12). This would not appear

to be the case for either the murine heart soluble EDTA-insensitive cPDE forms (band 1 = 246000 daltons compared to band 2 = 227000 daltons) or for the EGTA-sensitive cPDE enzymes of human uterine cytosols (band 1 = 229000 daltons compared to bands 3-7 = 175000 daltons).

Biochemical considerations.

My experiments based on nd PAGE coupled to the cPDE activity stain showed identical cPDE profiles for either cAMP or cGMP in all the tissue cytosols I studied. The staining intensity was similiar for both substrates when incubated with either 50 to 300 uM cAMP or cGMP. At lower substrate concentrations (10 uM), however, there was evidence for increased cPDE activity in the presence of 10 uM cGMP compared to 10 uM cAMP when I studied this effect in murine heart cytosols (Figs. 2:5e and 2:5f). It is necessary to reiterate, though, that no new cPDE activity bands were observed to appear at different substrate concentrations. This has important implications: 1. It is highly unlikely that there are discrete cPDE enzymes for the hydrolysis of either cAMP or cGMP. In other words, the catalytic binding site(s) probably reside(s) in the same enzyme. 2. It is unlikely that there are discrete high affinity and low affinity cPDE enzymes.

For the type I cPDE, which seemed to be the predominant soluble cPDE activity in the tissue cytosols I studied, there is controversy as to whether cAMP and cGMP are hydrolyzed by

separate catalytic sites as suggested by Geremia *et al.* (1984) or by a common catalytic site (Sullivan *et al.* 1987). I am, at present, unable to offer any definitive proof although my observations are consistent with a common catalytic site for both cAMP and cGMP with possibly a discrete high affinity cGMP site that becomes active at low substrate concentrations. Also, my findings tend to discredit the still popular practice of reporting high affinity cPDE and low affinity cPDE as if they were separate enzymes. This is usually done by studying the cPDE enzyme kinetics in crude extracts and then attributing the non-linear plots to low and high affinity enzymes (see Kofinas *et al.* 1987; Kurtz *et al.* 1987; Moses *et al.* 1987). My findings unequivocally demonstrated at least three or more cPDE forms in all the tissue cytosols I studied (see results of gradient nd PAGE on murine brain, kidney and liver Fig. 2:7) and these cPDE profiles were identical for each tissue cytosol from 50 uM (10 uM in murine heart) to 300 uM substrate concentrations. This multiplicity of both cPDE enzymes and isoenzymes makes it difficult to determine the relative contributions of the specific cPDE forms in crude enzymes or extracts. Therefore some form of separation technique of the cPDE forms, prior to attempting enzyme kinetics analysis, seems to be a necessary prerequisite. As I stated previously (Multiple Forms section in the Introduction), DEAE-cellulose anion-exchange chromatography has almost always been the method of choice for the separation of cPDE forms, on the basis of net protein charge, prior to further purification. My experience with this technique,

indicated that large losses of cPDE activity were incurred during the DEAE c.romatography stage in addition to the overnight dialysis and concentraion steps. Thus, it is not a suitable method for small tissue samples. The adaptation and modification of the method of nd PAGE coupled to the cPDE activity stain to slab gels, has enabled the separation of cPDE forms to be attained with very little soluble protein (0.1 to 0.5 mg soluble protein). Furthermore, several samples from different tissue cytosols can be assessed on a comparative basis for their cPDE enzymes. Also, by means of preparative nd PAGE, it is possible to excise the separated cPDE bands (note, in the absence of lead ions), elute them from the polyacrylamide gel and then calculate the kinetic parameters of these separated cPDE forms. Another important consideration of the nd PAGE method is the fact that there was likely to be less breakdown of the cPDE forms by proteolysis as: 1. The tissues were processed in the presence of the proteolytic inhibitor, PMSF. 2. The tissue cytosols were not exposed to excessive dilution as occurs with DEAE-anion exchange and dialysis. 3. The slab gel electrophoresis runs were performed at a very low current at 4° C in a period of only five hours.

It is interesting to note that, at no time did I detect any low molecular mass cPDE forms using the gel method (molecular masses > 162000 daltons). Thus, I did not detect the soluble cAMP-specific type IV cPDE of native molecular mass 60000 daltons (Thompson *et al.* 1979 (a)). This low molecular mass enzyme has also been described in the soluble fraction of human

lung (Moore and Schroedter 1982) although these latter authors speculated on the possibility of their enzyme being a catalytic subunit of a larger native enzyme. Of course, it cannot be entirely discounted that my gel method was not sensitive enough to detect the presence of this soluble type IV cPDE. When I performed sucrose ultracentrifugation (18 hour runs) on crude leiomyoma cytosols I detected three cPDE forms (Fig 5:4), one of which was a low molecular mass enzyme of 3.6 S (c.f. 46000 daltons). The seven forms that I detected, using the gel method, in leiomyoma cytosols, however, all had molecular masses of greater than 162000 daltons. It is also worth noting, based on my own experiences with sucrose gradient ultracentrifugation, that if leiomyoma supernatants, either crude or partially pure, were frozen and rethawed or stored for more than three weeks at - 70° C, lower molecular mass forms of cPDE were detected and regarded as proteolytic but active cPDE enzyme fragments.

Finally, the nd PAGE method might prove useful in the assessment of inhibitor/activator sensitivities of the cPDE activity forms of different tissues to a variety of compounds.

Clinical considerations.

From a clincal standpoint, I had hoped to be able to detect a soluble cPDE activity band that might be specific for tumour mammary cytosols. Indeed, the finding of two faster migrating cPDE forms in murine mammary tumour cytosols compared to other

normal murine tissue cytosols seemed promising. The work on human mammary cytosols also indicated two possible tumour associated cPDE forms with Rf ranges of 0.28-0.30 and 0.39-0.40. The subsequent observation of these two migrating cPDE forms in both normal myometrium and benign uterine tumours, however, suggested that these lower cPDE activity bands, might in fact, be type I cPDE isoenzymes associated with steroid hormone target tissues. An interesting finding, though, was the relatively reduced cPDE activity staining in leiomyoma cytosols compared to their normal myometrium cytosol counterparts. I have demonstrated the oestrogen and progesterone receptor content to be significantly higher in leiomyoma compared to myometrium ($p < 0.0002$) (Sadan *et al.* 1987). Therefore, there appears to be an inverse correlation between levels of oestrogen/progesterone receptors and the level of cPDE activity which is in agreement with the findings of Etingof *et al.* (1984). Also, this finding concurs well with that of Cho-Chung and associates (1978) who reported that cPDE activity was increased in regressing DBMA-induced rat mammary tumours. DMBA-induced tumours are known to posses high levels of functional oestrogen and progesterone receptors and regression of growth of these tumours is achieved by ovariectomy of the host which leads to withdrawal of steroid hormones and thus reduction of receptors. Of concern, therefore, is the fact that many breast cancer patients who have detectable levels of oestrogen/progesterone receptors in their tumours are often treated with the anti-oestrogen,

tamoxifen. This drug, however, has also been shown to be a type I cPDE inhibitor (Lam 1984) which would, at first, appear to be a disadvantageous effect in view of the fact that cPDE levels are observed to be increased in regressing DBMA-induced rat mammary tumours (Cho-Chung *et al.* 1978). Nonetheless, it is important to note that the levels of adenyl cyclase and cAMP are also increased in these regressing tumours (Cho-Chung *et al.* 1978). It seems, therefore, that despite the increased cPDE activity level, the overall concentration of cAMP is higher in regressing tumours. On the basis of this observation and assuming the increased cAMP levels to be associated with growth inhibition, it is interesting to speculate that tamoxifen in conjunction with either a drug that stimulates adenyl cyclase (a forskolin-like compound) or the use of site-specific cAMP analogues that have a much higher affinity for the cAMP receptor protein (Tagliaferri *et al.* 1988) might further increase the efficacy of tamoxifen in causing tumour regression. Alternatively, it should prove worthwhile to search for an anti-oestrogen that is inhibitory to all the cPDE types and is not solely specific for type I cPDE, as is the case with the calmodulin antagonist tamoxifen.

The assessment of the inhibitory effects of such an anti-oestrogen on soluble cPDE enzymes in mammary tumour cytosols could easily be performed with the nd PAGE technique coupled to the cPDE activity stain. The drug therapies proposed above might also be of value in the treatment of leiomyoma which are rapidly growing, steroid receptor positive benign tumours of the

uterus. This latter condition is usually treated surgically (Sadan *et al.* 1987).

Although I was unable to detect a specific tumour-associated soluble cPDE activity in mammary tissue cytosols, this was not the case for the nuclear-associated particulate cPDE in malignant mammary tissues (Chapter 3). This membrane-bound cPDE (only cAMP tested) was studied by using an ultracytochemical technique coupled to the cPDE activity stain. The nuclear-associated cPDE was strikingly observed in malignant mammary cells, to a lesser extent in fibrocystic mammary cells but was not apparent in normal human breast epithelial cells. This finding has important implications for possible therapeutic therapy against the mammary cancer cells. If the nuclear-associated cPDE could be characterized and a specific inhibitor identified then it might be possible to induce tumour regression by using the inhibitor to cause increased cellular cAMP concentration. This would be of particular value in those patients who have oestrogen/receptor negative tumours that are usually associated with more aggressive disease (Robinson et al. 1985). In short, anti-oestrogen therapy is ineffective against steroid receptor negative tumours and thus an inhibitor directed specifically against the nuclear-associated cPDE that I detected in all the malignant breast tumours, might be able to target those hormone-independent tumours. Of possible relevance to my findings is the recent report by Lichtner and associates (1987), who have demonstrated that the antimetastatic action of the pyrimido-pyrimidine analogue RA

233 (Rapenton) on tumour cell lines, is not merely the result of inhibition of platelet function but also the alteration in cAMP metabolism because of inhibition by RA 233 of type IV cPDE (Lichtner *et al.* 1987). It would be useful to perform further ultracytochemical experiments to establish whether this RA 233 inhibitor inhibits the nuclear-associated cPDE.

At present, I am studying a murine model which exhibits spontaneous hepatomas. There is sufficient normal liver for comparative studies between the normal liver and hepatoma tissues. I am using the methodology described in this thesis to study the soluble liver cPDE forms and I am developing new techniques to study the membrane-bound cPDE forms.

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